THE GLOBULINS OF THE GROUND NUT
(*Arachis Hypogaea*)

II. ELECTROPHORETIC EXAMINATION OF THE ARACHIN SYSTEM*

by

P. JOHNSON, E. M. SHOOTER, AND E. K. RIDEAL

Davy Faraday Research Laboratory of the Royal Institution, London (England)

I. INTRODUCTION AND EXPERIMENTAL

The ground nut globulins have been the subject of an electrophoretic examination by FONTAINE, IRVING AND WARNER[1]. To obtain sufficiently concentrated solutions for analysis at 0.5° C, these authors used an $NH_3.HCl$ buffer at p_H 9.26 and ionic strength 0.10 both for the extracting medium and as solvent buffer for the analyses. From the extract, which contained two major and small amounts of minor components, they prepared several fractions. Of interest in this investigation is an arachin fraction prepared by twice precipitating with 40% ammonium sulphate and a final precipitation from a solution in 10% sodium chloride by a tenfold dilution with distilled water. This fraction contained only the two major components whose mobilities were nearly equal. From the electrophoretic diagrams, FONTAINE *et al.* postulated that two components X and Y (A and B in the nomenclature of FONTAINE *et al.*) were present in the arachin fraction as an equilibrium system

$$X + Y \underset{k_2}{\overset{k_1}{\rightleftharpoons}} XY$$

where the reaction constants k_1 and k_2 were large and of the same order of magnitude.

An examination was also made of a protein fraction precipitated at p_H 4.5 by the addition of acid to a 10% sodium chloride extract. The electrophoretic patterns of this precipitate were similar to but not identical with those of the arachin fraction.

In Part I of this paper it has been confirmed that arachin is a reversibly dissociating molecule and that the molecular composition of an arachin solution is dependent upon the properties of the buffer solvent. Although $NH_3.HCl$ buffers were not investigated, by analogy with other systems, solutions in these buffers which have been dialysed for days would be expected to contain two components (parent molecule and dissociation products). The above experimental findings of FONTAINE *et al.* are not therefore unexpected, though our interpretation is not the same as theirs (see p. 385).

Because of the very limited solubility of fractions I and II in the p_H range 6.5 to 9.5

---

* For Part I, see *Biochim. Biophys. Acta*, 5 (1950) 361.

at 0°–4° C, the electrophoresis experiments have been conducted at 20° C. The limitations of field strength for convection-free electrophoresis at this temperature have already been discussed[3]. It has therefore been possible to examine and compare solutions of both fractions under exactly the same conditions of temperature, protein and salt concentration and time of solution as the previously reported experiments in the ultracentrifuge (Part I).

The electrophoresis apparatus was a Hilger instrument with a modified optical system, using a medium U tube equipped with a long centre section. The experimental manipulation of such instruments is described elsewhere. To ensure osmotic equilibrium between protein solutions and the buffer solvents (the importance of which is stressed on p. 392) dialysis for not less (and usually more) than 48 hours was necessary. For this reason electrophoretic examination is not very suitable for the investigation of half-life times of the longer equilibrium processes and is useless when these are less than 50 hours. However information of this type has been obtained where possible and its comparison with ultracentrifuge estimates is of considerable interest.

II. ELECTROPHORETIC ANALYSIS IN PHOSPHATE BUFFERS (I = 0.10)

a. *Solutions of fraction I*

In Fig. 1a, b and c are given respectively the electrophoresis diagrams (at approximately the same stage of resolution) of solutions of fraction I at p_H's 6.88, 7.08 and 7.94 whilst Fig. 2 contains graphs of mobility against p_H (dotted line).

p_H	$\Delta n \cdot 10^5$	Age of solution (hrs)
a. 6.88	105	50
b. 7.08	101	50
c. 7.94	126	120
d. 6.90	114	94

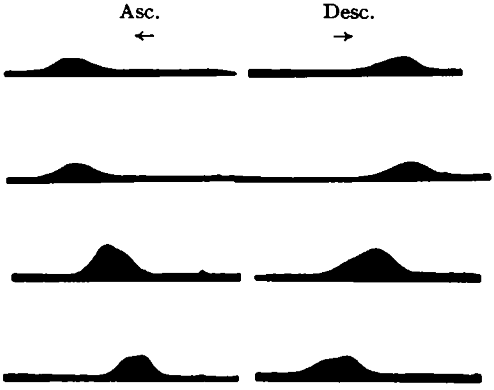
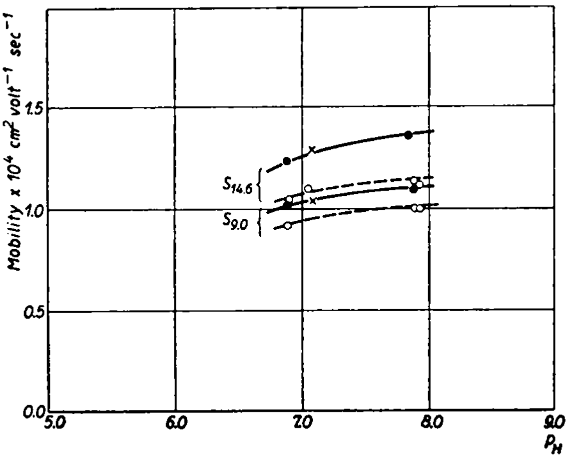


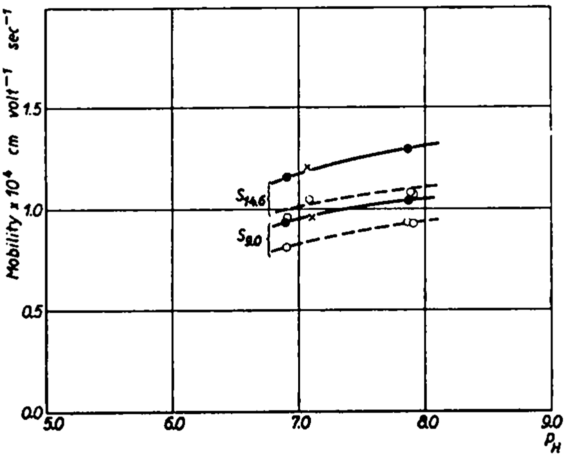
Fig. 1. Electrophoresis diagrams of solutions of fraction I in phosphate buffer; I = 0.10.

No complete separation of the two components which possess quite different molecular weights (330 000 and 180 000) and sedimentation constants was achieved at any p_H (cf. Fig. 4 Part I). The ascending boundaries which tended to be sharper than the descending (for reasons to be discussed later, p. 391) usually showed more clearly the possible presence of the two components but so incomplete was the separation that no assessment of relative concentrations or of small changes in concentration with p_H could be attempted.

The analysis given in line d. of Fig. 1—an excess of fraction I stirred with buffer—showed a different boundary contour on the ascending from the descending limb. It is



a.
Ascending boundaries



b.
Descending boundaries

Fig. 2. Mobility *vs* p_H graphs for fractions I and II in phosphate buffer; I = 0.10.

Fraction I: — — — — — —
Fraction II: ————————
Fraction I + 0.0025% $(NH_4)_2SO_4$: ×

thought that this is not to be interpreted as denoting very different concentrations of the two components in the two limbs but rather results from a combination of boundary anomalies and the incomplete separation of the components. The presence of boundary anomalies causes here a sharpening of the ascending and a blurring of the descending boundaries, the extent of the effect being determined by the osmotic contribution of the component forming the boundary. It is very unlikely therefore that final combined contours of the two incompletely separated boundaries will be the same on the ascending and descending sides. Other factors due to anomalies, *e.g.* change in apparent concentrations or change in relative mobilities of the two components will also differ within the boundaries and contribute to the alteration of the final contour (see p. 391).

It was possible to obtain better separation of the two components at higher field strengths but the disintegrating tendencies of thermal convection introduce uncertainty and preclude a detailed study.

b. *Solutions of fraction II*

In agreement with indications from the ultracentrifuge, solutions of this fraction were more nearly homogeneous (Fig. 3a and b).

The small amount of slow component (ca. 20% of the total at p_H 7.87) is actually dissociated $S_{9.0}$ species which arises

p_H	$\Delta n \cdot 10^5$	Age of solution (hrs)	Asc. ←	Desc. →
a. 6.90	100	40		
b. 7.87	97	100		

Fig. 3. Electrophoresis diagrams of solutions of fraction II in phosphate buffer; I = 0.10.

from the slow dissociation of the parent A_2 molecule under these conditions[3]. Values of mobility for the two components fall on the full lines of Fig. 2 rather than on the plots obtained with solutions of fraction I. It seems likely that solutions of fraction II when first prepared differed chemically from those of fraction I only in that they contained small quantities ($< 1\%$ saturation) of ammonium sulphate and it would seem possible that the observed increase in mobility could be explained as an increase in net negative charge due to absorption of sulphate ions.

To investigate this possibility a solution of fraction I in phosphate buffer was dialysed against phosphate buffer at p_H 7.08 containing 0.0025% of ammonium sulphate (10 moles/mole $S_{14.6}$ species). Although the electrophoretic patterns remained unaltered the mobilities of the two components fell almost exactly on the mobility graphs obtained with the solutions of pure fraction II (Fig. 2). Adsorption of sulphate ions must therefore occur and the adsorbed ions appear not to be removed by prolonged dialysis. It was further noted that even after precipitation of diluted solutions of fraction II (with an equal volume of buffer) at p_H 5.0 and subsequent re-solution and dialysis of the precipitate, the mobilities of the components remained at the high values given by the original fraction II. The adsorbed SO_4^{--} ions are not displaced by this type of precipitation which involves some dissociation of the parent species.

Whilst incomplete separation in electrophoresis precludes a quantitative correlation with ultracentrifuge results, it is to be noted that qualitatively the two sets of results are in agreement.

In estimating the rates of the equilibrium processes it is of interest to compare electrophoretic diagrams at different times for a given fraction and for the two fractions (Figs. 1 and 3) at a given time. The incomplete resolution of the two components makes estimates very approximate, but at p_H 7 it appears that the half-life times cannot be less than 50 hours and are probably greater, whilst at p_H 8 the half-lives are probably greater than 100 hours. More detailed information is available for the barbiturate system (p. 387).

c. *Calculations of Molecular Charge and of Bound Sulphate*

The two extreme models utilised in the calculation of the probable limits of the net charge of a protein molecule from its electrophoretic mobility are normally

a. a solvated sphere

and b. an unsolvated cylinder

Dissolved spherical protein molecules usually fall into the intermediate size category[4] for which the treatment of HENRY[5] is valid. Provided the terms $(z_i\varepsilon\psi)/kT$ are all $<< 1$, the net charge Q for such a molecule is given by

$$Q = \frac{4\pi\eta a\,(1 + \varkappa a + \varkappa v_i)}{f\,(\varkappa a)\,(1 + \varkappa v_i)} \qquad (1)$$

where a is the radius of the protein molecule

v_i is the average radius of the gegenions,

η is the solvent viscosity,

$\varkappa$ is the DEBYE-HÜCKEL function

$f(\varkappa a)$ is a function evaluated by HENRY

and u is the electrophoretic mobility.

This assumption—the normal DEBYE-HÜCKEL approximation—is severe, but several workers (*e.g.* ABRAMSON, MOYER AND GORIN[6]) have shown that net charges or valencies thus calculated agree well with the independent determinations from titration experiments up to valencies of ± 20. Therefore although the higher values of valency quoted in this section may be quantitatively uncertain their differences, which represent the numbers of sulphate ions bound, are probably more significant.

Since in the first instance the molecules are assumed spherical their radius 'a' may be derived from the SUTHERLAND-EINSTEIN equation

$$a = \frac{kT}{6\pi\eta D} \tag{2}$$

using the experimentally determined diffusion coefficient D. Thus the valency (γ) of the parent molecule ($S_{14.6}$ species), assuming the solvated spherical model, in phosphate buffers of ionic strength 0.10 becomes finally

$$\gamma = 32.25\, u.$$

The mobility of the descending fast boundary is strictly correct if, as is usual, the conductivity of the dialysed protein solution is used in its calculation. Columns 2 and 5 of Table I therefore contain the interpolated values of the descending mobility of the fast $S_{14.6}$ species in solutions of fraction I and II respectively whilst columns 3 and 6 contain the valency of this molecule (assumed a solvated sphere) determined from the above equation. The higher values in column 6 are due to the adsorption of sulphate ion.

TABLE I

VALENCY OF $S_{14.6}$ COMPONENT IN SOLUTIONS OF FRACTIONS I AND II. PHOSPHATE BUFFER; I = 0.10

	Fraction I			Fraction II				
1	2	3	4	5	6	7	8	9
pH	$u \cdot 10^4$	Valency		$u \cdot 10^4$	Valency		Increase in Valency due to adsorbed SO_4^{--} ions	
		Sphere 32.25 u	Rod 35.1 u		Sphere 32.25 u	Rod 35.1 u	Sphere	Rod
8.0	1.09	35.3	38.3	1.31	42.25	46.0	7.0	7.7
7.8	1.08	35.0	38.2	1.29	41.60	45.3	6.6	7.1
7.6	1.07	34.5	37.6	1.27	41.0	44.6	6.5	7.0
7.4	1.06	34.2	37.2	1.245	40.2	43.7	6.0	6.5
7.2	1.045	33.7	36.7	1.22	39.9	42.8	5.7	6.1
7.0	1.015	32.7	35.6	1.175	37.9	41.2	5.2	5.6
6.8	0.95	30.6	33.4	1.12	36.1	39.3	5.5	5.9

All values of valency are -ve

For model b, the unsolvated cylinder, a similar procedure is followed using instead of the actual radius, the radius of a hypothetical unsolvated sphere a_0 given by

$$a_0 = \left(\frac{3M\bar{v}}{4\pi N}\right)^{1/3} \tag{3}$$

where M is the molecular weight of the protein, $\bar{v}$ is the partial specific volume and N the AVOGADRO Number. Thus again for the parent $S_{14.6}$ component (assumed an unsolvated sphere)

$$\gamma = 22.5\ u.$$

The experimentally determined frictional ratio f/f_0 of 1.24 for this component leads to an axial ratio $\left(\frac{a}{b}\right)$ of 4.7 if the molecule is assumed unsolvated. It is then possible from calculations of GORIN[7] to determine the valency of this unsolvated cylinder.

Thus

$$\gamma = \frac{22.5}{0.64}\, u = 35.1\ u$$

where 0.64 (= R) is the ratio of the mobilities of the unsolvated cylinder of axial ratio 4.7 and the unsolvated sphere of radius a_0 at an ionic strength of 0.10. The values of valency for this model are found in columns 4 and 7 and it is to be noted that, although larger, these do not differ fundamentally from those for the previous model.

It is most probable that the actual valency of the parent molecule lies between those determined for the solvated sphere (3 and 6) and the unsolvated cylinder (4 and 7). Of considerable interest are the increases in valency of the parent molecule due to adsorption of sulphate ion (columns 8 and 9). The decrease observed in these values at the lower p_H's is probably not significant being dependent on the relative slopes of the mobility-p_H plots. Thus 3 or 4 sulphate ions are bound per molecule of the $S_{14.6}$ species over the p_H range 6.90–8.00 and these are not removed by dialysis or precipitation of the parent molecule at the isoelectric point.

No attempt has been made to repeat these calculations for the sub-molecule ($S_{9.0}$). The mobility of the slower component of a two component mixture when calculated in the normal manner is incorrect by a factor dependent on the magnitude of the boundary anomalies. Although some calculations are made later of the effect of boundary anomalies, a reliable value for the mobility of the $S_{9.0}$ species cannot be obtained simply from present experimental data.

d. *The equilibrium reaction as a function of the charge of the molecules*

From the ultracentrifuge results it is most probable that the parent molecule dissociates into half molecules of greater asymmetry. The influence of the two factors a. shape and b. size on the migration of the half molecules may be briefly discussed.

a. The mobilities of both parent (u_{A_2}) and half molecules (u_A) may be compared with the mobilities of spheres of equal molecular volume and charge ($u^0_{A_2}$ and u^0_A resp.) with the aid of the calculations of GORIN. Thus at an ionic strength of 0.10 and assuming axial ratios of 2.6 and 4.6

$$\frac{u_{A_2}}{u^0_{A_2}} = 0.75 \text{ and } \frac{u_A}{u^0_A} = 0.65$$

The more asymmetric sub molecule suffers the greater retardation.

Combining we have

$$\frac{u_A}{u_{A_2}} = \frac{0.65}{0.75} \times \frac{u^0_A}{u_{A_2}}$$

References p. 396.

b. On the other hand the smaller spherical molecule will (for a charge equal to that of the parent molecule) move faster than the parent molecule by a factor given by eqn. (1) and equal to

$$\frac{u^0_A}{u^0_{A_2}} = \frac{(1 + \kappa a + xv_i)_{A_2}}{(1 + \kappa a + \kappa v_i)_A} \cdot \frac{f(\kappa a)_A}{f(\kappa a)_{A_2}} \approx 1.47$$

Thus

$$\frac{u_A}{u_{A_2}} = \frac{u^0_A}{u^0_{A_2}} \times \frac{0.65}{0.75} \approx 1.47 \times \frac{0.65}{0.75} \approx 1.26$$

represents the ratio of the mobilities of the half and parent molecules assuming equal charge, only changes in size and shape occurring in the dissociation having been taken into consideration. If the charge on A is half that on A_2 and no other factor intervenes, the mobility of the half molecule would therefore be expected to be just over half the mobility of the parent molecule and a very good separation of these two components would be achieved in the electrophoresis experiments.

The experimental observation that the mobility of the sub-molecule is very considerably higher than half the mobility of the parent molecule may arise from either or both the following:

a. inadequate theoretical treatment of the effect of size and asymmetry on electrophoretic migration

b. an increase in the net negative charge of the half molecule after dissociation *i.e.* the appearance of negative or disappearance of positive groups.

It is probable that a. is only of secondary importance since it has already been mentioned that good agreement has been found for valencies calculated independently from electrophoretic and titration data.

The second consideration implies that the reverse process, association, must then involve a disappearance of negative or appearance of positive groups. However, the presence of sulphate ions which increase the net negative charge, tends to retard dissociation (or significantly alter the equilibrium position) and the role played by sulphate ions and the importance of charge effects in general on the equilibrium processes is not at all clear.

III. EXAMINATION OF THE GROUND NUT FRACTIONS IN BARBITURATE BUFFERS ($I = 0.04$)

The incomplete electrophoretic separations in phosphate buffers made quantitative measurements on the dissociation of the $S_{14.6}$ species impossible. It was hoped, however, that the known property of the barbiturate ion in allowing better electrophoretic separations (*e.g.* SVENSSON[8]) would also operate in the ground nut system. In addition (for negatively charged protein molecules) boundary anomalies are smaller in this particular buffer and the final interpretation of the schlieren diagrams is much simplified.

a. Solutions of fraction I

The electrophoretic diagrams for these solutions (p_H's 7.00–9.50) are given in Fig. 4 and the corresponding mobility graphs in Fig. 5 (open circles).

It is first apparent that an increase in the concentration of the faster migrating component in both ascending and descending boundaries occurs with decreasing p_H. By comparison with ultracentrifuge results this component is most probably then to be identified with the parent $S_{14.6}$ component.

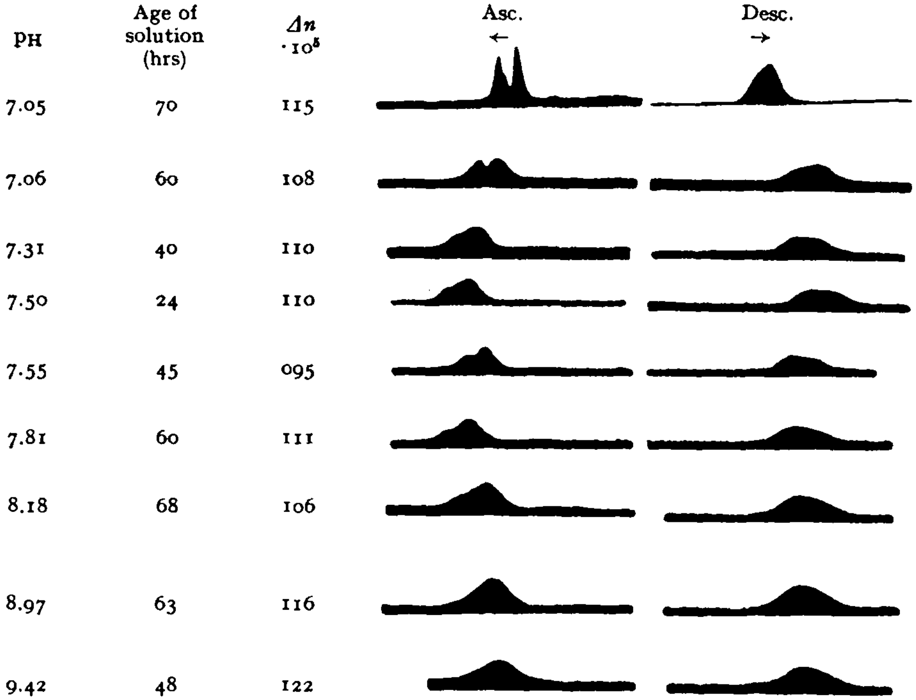
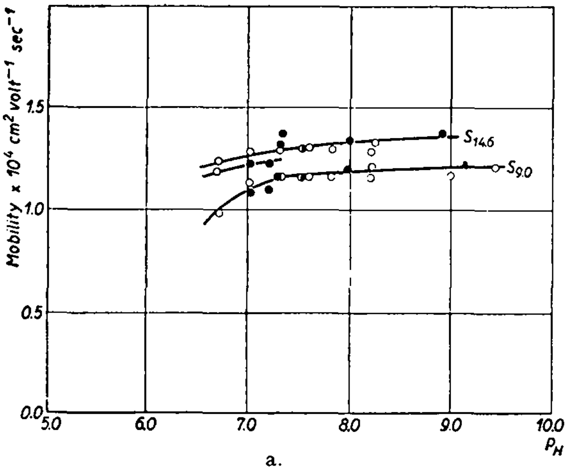


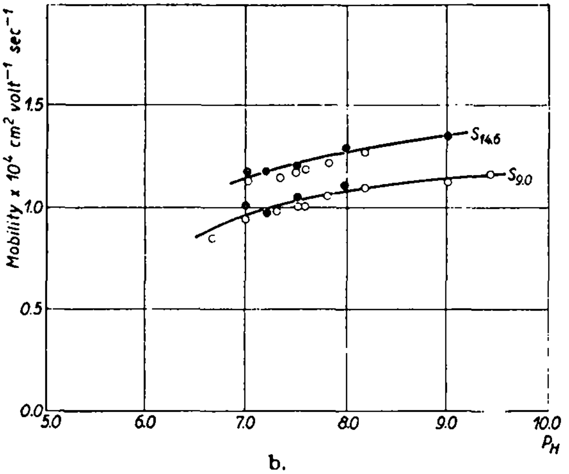
Fig. 4. Electrophoresis diagrams of solutions of fraction I in barbiturate buffers; I = 0.04

At the higher p_H's of 8.97 and 9.42 the boundaries were composed mainly of the other slow migrating, $S_{9.0}$, component whilst at the lower p_H's the amount of $S_{14.6}$ component visibly increased. In view of the slowness of reaction rates at these p_H's (see Part I), it is unlikely that the relevant electrophoretic diagrams correspond with completely equilibrated systems, but the general observation above is not in doubt. Apart from the ascending boundary at p_H 7.0 the separation of the $S_{14.6}$ and $S_{9.0}$ species was again incomplete and no accurate area determinations for individual components could again be obtained.

It will be recalled that ultracentrifuge results indicate that at the higher p_H's an equilibrium solution contains some 80% of dissociated $S_{9.0}$ species, this quantity decreasing at the lower p_H's until below p_H 7.0 the parent $S_{14.6}$ species predominates. Thus there is qualitative agreement between the two sets of results. At p_H 7.0 the separation of the components in the ascending limb was good and the relative concentrations corresponded approximately with those of the known equilibrium position at this p_H, as determined by the ultracentrifuge, *i.e.* a slightly higher proportion of the dissociated $S_{9.0}$ species. As anticipated from the results of Part I, the relative concentrations did not alter with the time of solution, the equilibrium having been established within the time of the shortest period of dialysis at p_H 7.0 (*i.e.* within 45 hours, Fig. 6).



a.
Ascending boundaries



b.
Descending boundaries

Fig. 5. Mobilities of parent and dissociated molecules of ground nut globulin in barbiturate buffers; I = 0.04.

The concentrations corresponding to the ascending boundaries in Fig. 6 are given in Tab.e II.

Although the descending boundary at p_H 7.0 (Fig. 6) appeared to give the reverse distribution of concentrations (*i.e.* a greater proportion of the faster component) it is again felt (cf. p. 378) that the anomalous spreading of individual boundaries previously referred to, particularly when mobilities are alike and separation incomplete, may mask the true composition of these migrating boundaries. It may be noted, in this connection, that the increase in the concentration of the fast ($S_{14.6}$) component with decreasing p_H seems to occur more rapidly in the descending than in the ascending limb. This effect, again, must be due to the unequal spreading of the various boundaries. It cannot arise as a result of the equilibrium reactions since the half life is always longer (and at high p_H very much longer) than the duration of an experiment.

The results of FONTAINE *et al.*[1] may be conveniently discussed at this point. Their preparation of arachin originated from an $NH_3.HCl$ extract at p_H 9.26 and ionic strength 0.10 and the state of association of the protein in this solution is not

	Age of solution (hrs)	$\Delta n \cdot 10^5$
a.	45	108
b.	60	108
c.	60	117
d.	85	115

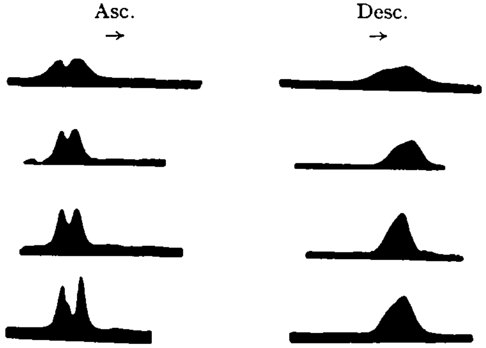


Fig. 6. Electrophoresis diagrams of solutions of fraction I in barbiturate buffer; p_H 7.05–7.08, I = 0.04

References p. 396.

TABLE II

RELATIVE CONCENTRATIONS (APPARENT) OF THE TWO COMPONENTS (FROM ASCENDING BOUNDARY); PH'S NEAR 7.0 IN BARBITURATE BUFFER, I = 0.04

Experiment of Fig. 22	PH	Age of solution (hrs)	Relative conc. %	
			$S_{14.6}$	$S_{8.5}$
a	7.08	45	41	59 (± 3)
b	7.06	60	42	58
c	7.05	60	48	52
d	7.05	85	46	54
Ultracentrifuge result			45	55

accurately known. However from other results it seems likely that the equilibrium composition under these conditions contains a considerable amount of the dissociated form and the appearance in electrophoresis of two partly separated boundaries in this buffer is not therefore unexpected. An analysis of this fraction at 20° C (Fig. 7) did not show the presence of two separate boundaries but increased rates of diffusion at the higher temperature could effectively mask the separation.

Temp. °C	PH	$\Delta n \cdot 10^5$	F Volts/cm
20	9.56	179	2.02
0.5	9.56	(by FONTAINE)	

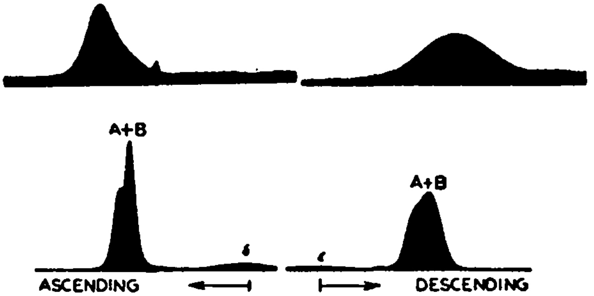


Fig. 7. Electrophoresis diagrams of FONTAINE's arachin in NH_3.HCl buffer; I = 0.10

On the other hand the results of FONTAINE *et al.* may conceivably represent some fine structure in the parent molecule which is apparent only at temperatures below 20° C. The apparently different concentration distributions obtained from the incompletely resolved boundaries in the two limbs of the U-tube are probably caused by boundary anomaly effects of the type already discussed (p. 378) and it is not necessary to invoke the interaction of two chemically different proteins. The latter explanation would be difficult to reconcile with ultracentrifuge results which indicate that the arachin fractions prepared according to FONTAINE *et al.* consist of one chemically defined type of protein molecule with variable amounts of dissociation product only.

In the last of the experiments recorded at p_H 7.0 (Fig. 6d) the faster component split into two to give an additional intermediate component. The latter (which did not arise by convection) could very easily remain undetected where the initial separation of the two main boundaries was not so sharp as in the above experiment (*e.g.* Fig. 6a, b or c). The component was not detected in the ultracentrifuge experiments and cannot therefore be a further dissociation product of the $S_{9.0}$ species. Its possible significance is discussed later.

b. *Solutions of fraction II*

The electrophoresis patterns are presented in Fig. 8 and the mobilities plotted (full circles) in Fig. 5.

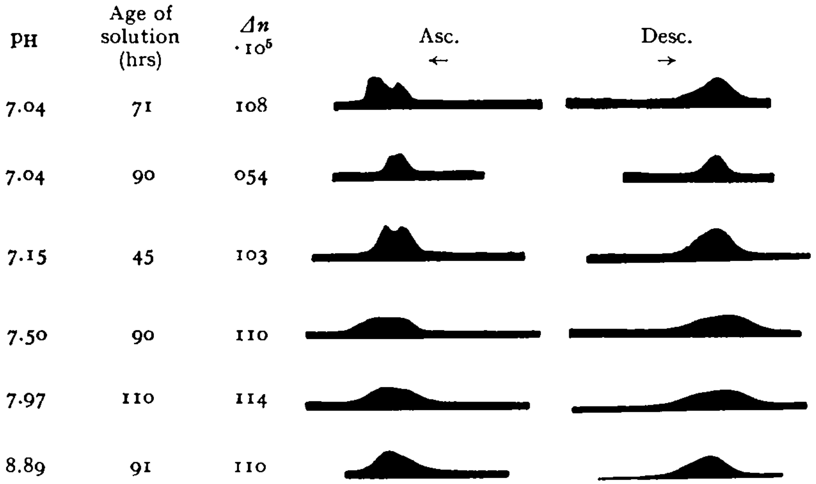


Fig. 8. Electrophoresis diagrams of solutions of fraction II in barbiturate buffers; I = 0.04

It is apparent that the mobilities of the two components in solutions of fraction II at the various p_H's are within experimental error identical with the mobilities of the components in solutions of fraction I and correspond therefore to parent and dissociated forms of the protein. It will be recalled that the mobilities of the components in phosphate solutions of fraction II were higher than those of fraction I probably because of the adsorption of sulphate ions. The identity of the above mobilities in the barbiturate buffers must indicate that the sulphate ions are replaced on the surface of the protein by the barbiturate ions. In addition, this replacement affects the rates of the two opposing reactions in as much as these rates are measurable at all p_H's within the barbiturate buffer range whereas they are exceedingly slow in the phosphate-sulphate ion system at p_H 8.0, and slower still at higher p_H's.

The analysis at p_H 8.89 after 91 hours gave a main component whose mobility corresponded with the faster component of fraction I and could therefore be identified with the $S_{14.6}$ species. This is completely confirmed when the ultracentrifugal result is recalled, for this solution contained on analysis after 198 hours approximately 90% of the $S_{14.6}$ species. The concentration of the dissociated, slow electrophoretic component is probably less than 10–15% and emphasises the slow rate of dissociation at these higher p_H's.

The rates of equilibration were shown to increase at lower p_H's and the analyses at p_H 7.97 and 7.50 after similar times of solution confirmed the presence of larger quantities of the dissociated material.

Analysis around p_H 7.0. The separation achieved in the ascending boundary at

p_H 7.15 was similar to that obtained in solutions of fraction I at p_H's near 7.0. About equal concentrations of the two forms were present in this solution of fraction II which had remained at this p_H for only 50 hours. Its composition achieved after 45 hours differed only slightly from the equilibrium composition at p_H 7.05 (Fig. 6). Solutions of fraction I it will be recalled equilibrated within 45 hours at p_H 7.05.

An attempted experiment at p_H 7.04 with fraction II after solution for 50 hours failed unexpectedly because a precipitate formed in the ascending boundary when the field strength was applied. Although the precipitate gradually disappeared during the course of the experiment the boundary became broken and relative concentrations uncertain (Fig. 8). However the addition to this solution of an equal volume of buffer (final $\Delta n = 0.00054$) and further standing for 16 hours did produce a composition (Fig. 8) very similar to that of the known equilibrium state at this p_H. This point is further discussed later (p. 389).

Comparing electrophoretic diagrams under given conditions for fractions I and II it is seen that at the lower p_H values (< 7.5) where reaction rates are relatively rapid compared with dialysis times, approximately the same equilibrium position is indicated, but, at higher p_H's, differences occur which on the basis of Part I must be attributed to insufficient equilibration times. Table III contains a comparison of half-life times as estimated by electrophoretic and ultracentrifuge examination. Within experimental error the whole of the electrophoretic data are in accordance with the views on the dissociation system developed in Part I.

TABLE III

HALF-LIVES OF THE EQUILIBRIUM REACTIONS IN BARBITURATE BUFFER; $I = 0.04$

p_H	Half-life (hrs)	
	Ultracentrifuge results	Electrophoresis results
9.0	> 200	$\gg 90$
8.0	slightly < 200	100–200
7.56		≈ 100
7.30	< 100	
7.15		≈ 50
7.00	(≈ 10)	

c. *Electrophoretic fractionation* was possible only for the ascending boundary in the barbiturate system at p_H 7.0. The original four-section U-tube of TISELIUS was used and a very small quantity 0.3 ml (at low concentration) of the component producing the faster ascending boundary (already identified as the $S_{14.6}$ species) was collected. The sedimentation diagram of the extracted solution taken one hour after removal from the cell showed only one very broad peak (protein conc. $< 0.2\%$). Although the peak was small its rapid spreading during sedimentation suggested the presence of more than one component. The mean sedimentation constant was 13.3 ± 0.5 S. It would therefore appear that the solution contained already some quantity of the dissociated $S_{9.0}$ species —a result which confirms the fairly rapid rate of equilibration at p_H 7.0.

d. *Calculation of the charge of the parent molecule in barbiturate buffers*

Similar calculations as on p. 379 lead to the net charge of the parent molecule in these buffers at I = 0.04. The equations for the two models are

a. solved sphere $\gamma = 25.03\ u$
b. unsolvated cylinder $\gamma = 26.7\ u$

and the values of the valency are quoted in Table IV.

TABLE IV

VALENCY OF $S_{14.6}$ IN BARBITURATE BUFFER; I = 0.04

PH	u (Desc.) $\cdot 10^4$	Valency (-ve)	
		Sphere 25.03 u	Rod 26.7 u
9.00	1.33	33.3	35.6
8.75	1.32	33.05	35.3
8.50	1.30	32.50	34.7
8.25	1.28	32.0	34.2
8.00	1.26	31.6	33.6
7.75	1.23	30.8	32.8
7.50	1.20	30.1	32.1
7.25	1.19	29.8	31.8
7.00	1.17	29.3	31.2

IV. ELECTROPHORETIC ANOMALIES

As yet in all the comparisons of electrophoretic and ultracentrifuge data no detailed allowance has been made for the effect of electrophoretic anomalies on the mobilities and relative concentrations of the two components. These anomalies are discussed fully later but, anticipating further results, under the normal conditions of analysis used in this investigation, apparent concentrations do not differ from true concentrations by more than 10%. On the other hand, it has not been possible here to estimate concentrations to an accuracy greater than 10–15% in the case of sedimentation diagrams or about 10% in electrophoresis. Conclusions already reached involve larger effects and are therefore not invalidated by the additional presence of boundary anomalies but it is to be noted that the calculations outlined later were performed before this conclusion was reached.

a. *Experimental investigation of the anomalies in the present system*

LONGSWORTH[9] showed that the true composition of a mixture may be obtained by extrapolation of the apparent composition either to zero protein concentration at constant ionic strength or to infinite salt concentration at constant protein concentration.

For several reasons the latter method is impracticable and it was decided to apply the former at p_H 7 in barbiturate buffer on the ascending boundaries. It has been shown in the ultracentrifuge that variation of protein concentration did not affect the relative quantities of the two components which justified the attempt.

Surprisingly, however, with increasing dilution of protein a very considerable decrease in the apparent concentration of the slower component occurs in the ascending limb until at the highest dilution precipitation occurs (Fig. 9).

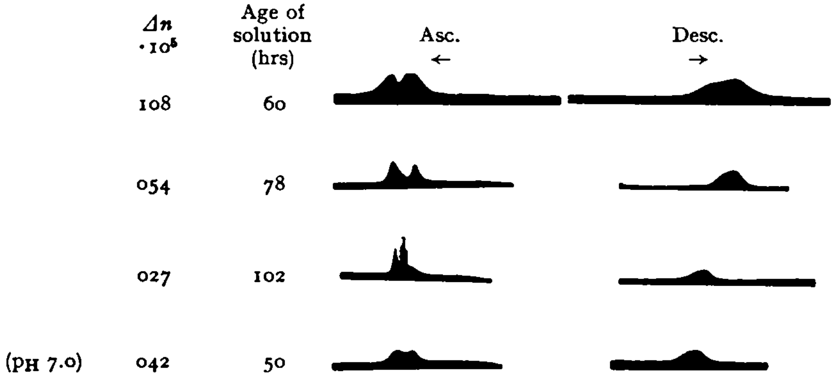


Fig. 9. Effect of dilution of protein. Electrophoretic diagrams of solutions of fraction I in barbiturate buffer; $p_H = 7.06$, $I = 0.04$

Little change can be detected in the outline of the descending boundary. Not only is the change in the ascending limb in the opposite sense to that required by boundary anomaly effects but it is also dependent on time. Thus at the concentration corresponding to the refractive index change $\Delta n = 0.00079$ an additional and gradual transformation of the slow to the fast component with time was detected, probably proceeding to equilibrium (Fig. 10).

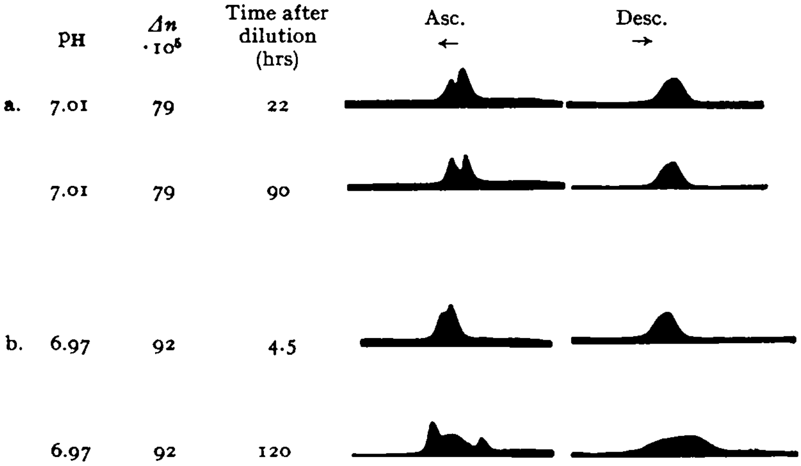


Fig. 10. Effect of time on the electrophoresis diagrams of the ascending boundaries in barbiturate buffer; $p_H = 7.0$, $I = 0.04$

The apparent percentage compositions of the diluted solutions are quoted in Table V and the ascending mobilities plotted against Δn in Fig. 11.

TABLE V

APPARENT COMPOSITION FROM THE ASCENDING BOUNDARY IN BARBITURATE BUFFER; $I = 0.04$, $p_H = 7.06$

Δn	Age of solution (hrs)	% composition	
		$S_{14.6}$	$S_{9.0}$
0.00108	—	42	58
0.00092	120	42	58
0.00079	90	50	50
0.00054	40	61	39
(0.00042	18	58	42)

It is to be noted that under identical conditions in the ultracentrifuge no change between the relative proportions of $S_{14.6}$ and $S_{9.0}$ was observed (*e.g.* Fig. 7 Part I) so that the changes of Figs. 9 and 10 appear peculiar to electrophoresis. They cannot merely be anomaly effects in view of the identity of the conditions at the different times.

It is significant that the change of composition with dilution and time occurs in the limb where reasonably clear separation of the two components is achieved (it will be stressed later that this separation is an artefact of the electrophoretic anomalies which cause artificial sharpening of the ascending boundaries). It may then be possible that this separation allows some type of chemical reaction to proceed in this limb. Such a reaction, detected in electrophoresis experiments but not in the ultracentrifuge must involve a change in net charge and it is of interest to enquire how this might arise.

The interaction of the protein molecules with barbiturate ions might be represented

$$A_2 + xB^- \rightleftharpoons (A_2B_x)^{-m}$$

$$A + yB^- \rightleftharpoons (AB_y)^{-n}$$

The mobilities and concentractions of the two complexes are actually measured in the electrophoresis experiments. By acquiring additional barbiturate ions either from solution or from the complex $(A_2B_x)^{-m}$ the slower complex could achieve the mobility of the faster, *e.g.*

$$(A\ B_y)^{-n} + zB^- \rightleftharpoons (A\ B_{y+z})^{-m}$$

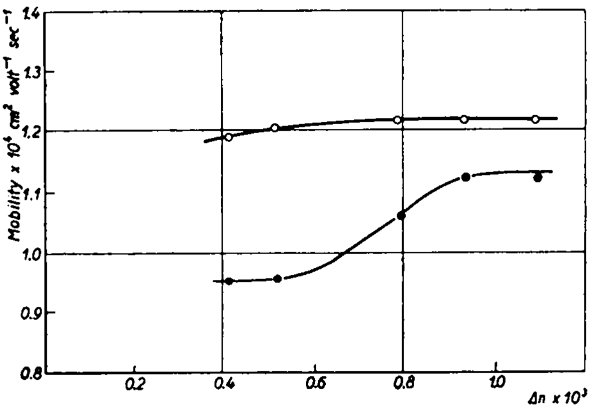


Fig. 11. Effect of dilution on the mobilities of the ascending boundaries given in Fig. 9 and 10. Barbiturate buffer; $p_H \approx 7.00$, $I = 0.04$

References p. 396.

Some such type of reaction might account for the changes in composition observed only in the ascending boundaries.

In some previous experiments at p_H 7.0 a third boundary of mobility intermediate between the two main boundaries appeared. This boundary would be present if an intermediate complex existed.

Thus it is not possible to test electrophoretic anomaly theory in regard to relative concentrations but the plot of mobility against protein concentration is of considerable significance in this connection, and is considered later.

LONGSWORTH[9] has demonstrated that the moving boundaries in electrophoresis experiments are subject to several anomaly effects. The conductivity effect usually causes artificial sharpening of ascending and artificial blurring of descending boundaries whilst at p_H's above the isoelectric point of the protein these processes are opposed by the p_H effect, the magnitude of the latter being dependent upon the slope of the mobility *vs* p_H graphs. Since, for the ground nut system, this slope is usually small (Fig. 5) the conductivity effect considerably outweighs the p_H effect at all p_H's.

In addition DOLE[10] has been able to show that all deviations from ideal behaviour increase as the relative mobility (r) of the buffer ion, of the same sign as the protein ion, increases. Thus, for a negatively charged protein, smaller deviations will be obtained in barbiturate (B^-) buffers ($r_{Na^+} = + 1.000$; $r_{B^-} = - 0.464$) than in chloride buffers ($r_{Cl^-} = - 1.681$).

b. *The extent of the anomalies in the present electrophoretic work*

The actual compositions of the buffers used are given in Fig. 12.

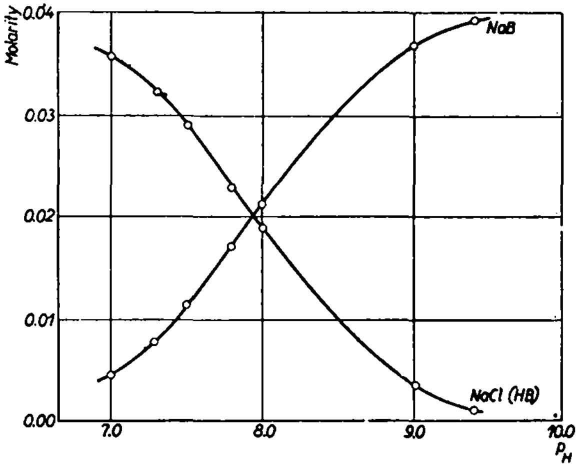


Fig. 12. Composition of barbiturate buffers

NaB = Sodium diethylbarbiturate
HB = Diethylbarbituric acid

According to LONGSWORTH AND DOLE the anomalous effects should increase as the ratio $\frac{[Cl^-]}{[B^-]}$ increases (*i.e.* with decreasing p_H) and become very large at p_H 7.0. If the mobilities of the fast and slow boundaries in the two limbs are examined it is evident (Fig. 13) that whilst at the higher p_H's the mobilities of the corresponding descending and ascending boundaries are almost identical, considerable divergence occurs at the lower p_H's.

The differences in mobility at the lower p_H's therefore indicate the increasing anomalous effects to be associated with buffers containing a preponderance of the fast Cl^- ion. The presence of anomalies at p_H 7 is also shown by Fig. 11 which demonstrates the changing relative mobilities of the two components in the ascending boundary with changing protein concentration. Thus at the higher protein concentrations the presence of larger anomalies causes a marked increase in the apparent mobility of the slower component, though the fast component is not so much affected. On the other hand at the lower protein concentrations the apparent mobility values for both components are not very different from those reported in Fig. 5 for the more reliable descending boundary.

In parallel with the above differences in mobility, ascending boundaries will tend to be sharper than the descending (for $r_B : r_{\text{Protein}} = 1:0.515$) and the effect becomes more pronounced at the lower p_H's. Whilst at p_H's above 8.0 the ascending and descending boundaries are very similar in diffuseness (Fig. 14a) (an indication that the conductivity effect and anomalies are in general small), at p_H 7.0 the sharpening of the ascending boundary makes possible a clear separation of the two components.

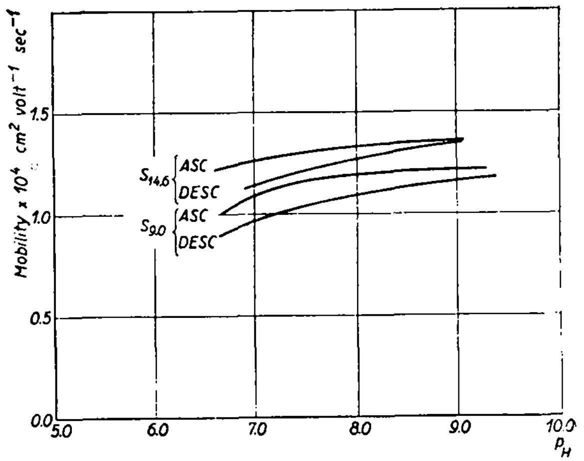


Fig. 13. Mobility *vs* p_H graphs for solutions of fractions I and II in barbiturate buffer; I = 0.04

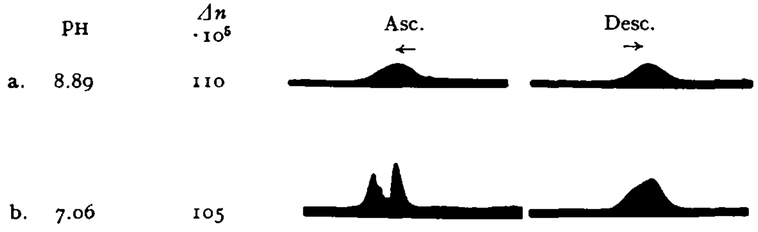


Fig. 14. Effect of boundary anomalies on electrophoresis diagrams

The artificial blurring on the descending side at this same p_H prevents a similar separation.

To decrease the extent of the anomalous effects at this p_H and ionic strength in order to test the above explanation it would be necessary to increase the ratio $\frac{[\text{NaB}]}{[\text{NaCl}]}$ keeping the buffer ratio $\frac{[\text{NaB}]}{[\text{HB}]}$ constant. It is not however possible to increase appreciably the molarity of the NaB and also of the HB because of the limited solubility at 20° C of the undissociated acid.

It can be shown, however, that the sharpness and clarity of separation of the ascending boundaries at these p_H's is very sensitive to changes in conductivity (and salt concentration) of the protein solution. Thus whilst a normal experiment on a dialysed solution of fraction II at p_H 7.33 gave the normal diagrams of Fig. 15a, a similar protein solution in incomplete osmotic equilibrium with the buffer through slight evaporation gave the very different diagrams of Fig. 15b.

Since small changes in conductivity have such striking effects on the electrophoretic diagrams it is clearly essential that complete osmotic equilibrium between protein and buffer solutions be established.

	Specific Conductivity				
	Δn	Protein solution	Buffer solution	Asc. ←	Desc. →
a. Normal dialysed solution	0.00083	0.00359	0.00360		
b. Incompletely dialysed solution	0.00085	0.00368	0.00360		

Fig. 15. Complete and incomplete equilibration (by dialysis) of protein and buffer solutions. Fraction II in barbiturate buffer; $p_H = 7.33$, $I = 0.04$

c. Calculation of anomaly effects and apparent composition

The calculations of valency for the $S_{14.6}$ species already outlined gave a mean value in barbiturate buffer at $I = 0.04$ and p_H 7.0 of -30.25. The charge may now be expressed as $\frac{-30.25}{330\,000} = -0.915 \cdot 10^{-4}$ faraday equivalents per gram and the concentrations of protein at this p_H in equivalents. In the following calculations using the method of DOLE, the mobility of the $S_{14.6}$ component has been taken from the descending fast boundary which gives correct mobilities. The mobility of the slow component is not determined in any of the electrophoresis experiments (except possibly at the higher p_H's were the solutions are almost monodisperse with respect to the $S_{9.0}$ species) and it is therefore necessary to examine the detailed anomalous effects over a range of mobility for this component. The calculations have been performed for a typical mixture, *i.e.* 0.3% of both the $S_{14.6}$ and $S_{9.0}$ species in 0.04 *M* solutions of NaCl (full lines) and NaB (dotted lines) at p_H 7.0, and the apparent theoretical composition given by the electrophoretic analysis is plotted against the ratio of the mobilities of the $S_{9.0}$ and $S_{14.6}$ species from the value 0.50 to 1.0 (Fig. 16).

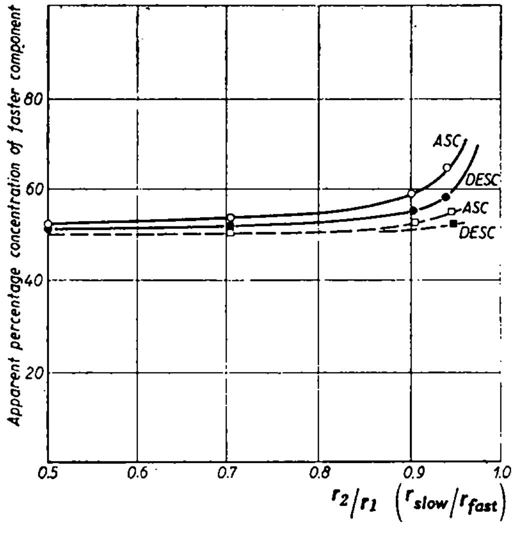


Fig. 16. Calculations of apparent composition by the theory of DOLE. 0.3% each of parent and dissociated molecule in 0.04 *M* solutions of NaCl (full lines) and NaB (dotted lines) at p_H 7.0.

The deviations of apparent from the actual composition (50% $S_{14.6}$, 50% $S_{9.0}$) are greater in the NaCl solution. Ascending boundaries are most affected but the deviations increase in both limbs as the mobilities of the two components become closer. The maximum effect of the anomalies in the barbiturate experiments may now be calculated.

The descending mobilities of the two components at p_H 7.0 are 1.13 and $0.96 \cdot 10^{-4}$ cm^2 volt^{-1} sec^{-1} resp. The latter mobility may be corrected approx. for the higher value of conductivity of the solution between the fast and slow boundaries by the factor 1.02. The ratio of the slow to fast mobility then becomes 0.87 : 1.00. From Fig. 16 the corresponding apparent percentages of the fast component in the sodium chloride solutions are 58% (Asc.) and 56% (Desc.) and in the sodium barbiturate solution 52% (Asc.) and ca. 50% (Desc.). The values of apparent composition in the *actual* barbiturate-NaCl buffer at p_H 7.0 will be slightly less than those quoted for the sodium chloride solution, whilst at p_H 9.0 the deviations will be smaller and of the same order as determined for the sodium barbiturate solution.

These maximum deviations are considerable at p_H 7.0, but it must again be stressed that at the low concentrations employed in this investigation an actual area of a schlieren peak cannot be determined with an accuracy much greater than 10%. The previous comparisons of electrophoretic and ultracentrifugal data have been made with this degree of accuracy in mind. At higher p_H's than 7.0 the deviations become less than this experimental error.

Calculations by the theory of HOCH[11]

These calculations have been made for a two component system, K^* the proportionality factor being taken as equal to the experimentally determined value for serum albumin in phosphate buffer, I = 0.10 *i.e.* $K = 0.06$. The total concentration of the protein mixture was, as above, 0.6% and the three series of computations were made for the following actual compositions, (i) 95% fast, 5% slow; (ii) 50% fast, 50% slow; (iii) 5% fast, 95% slow; Fig. 17 gives apparent composition as a function of r_2/r_1.

Thus the apparent concentration of the faster component only approaches that of the total mixture when the mobility of the slower component is practically equal to that of the faster *i.e.* differs by not more than 2%. Normally a difference of mobility of 2% is undetectable and the extreme deviations of this theory have no practical significance. The deviations of composition for the ground nut system (curve 2, Fig. 17, shaded area) amount to 5%—within experimental error—and it is noticed in general that this curve (2) coincides approximately with the computations on the system in 0.04 M sodium chloride using the theory of DOLE (Table VI).

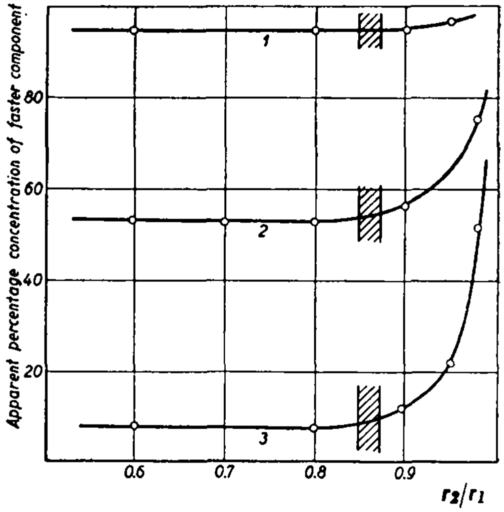


Fig. 17. Calculations of apparent composition by the theory of HOCH. Total concentration 0.6%, $K_1 = K_2 = 0.06$.

Curve 1. Actual composition 95% fast, 5% slow
Curve 2. Actual composition 50% fast, 50% slow
Curve 3. Actual composition 5% fast, 95% slow
Shaded area represents range of present mobilities

---

* For definition of K see original paper by HOCH[11]

TABLE VI

COMPUTATIONS BY THE THEORIES OF DOLE AND HOCH. MIXTURE CONTAINS 0.3% OF $S_{14.6}$ AND 0.3% OF $S_{9.0}$ COMPONENTS; pH 7.0

$\frac{r_2}{r_1}$	DOLE Apparent conc. of $S_{14.6}$ fast component in 0.04 *M* NaCl		HOCH Apparent conc. of $S_{14.6}$ fast component; $K = 0.06$	
	Asc.	Desc.	Asc.	Desc.
0.6	52.8	51.8	52.4	52.4
0.7	53.8	52.0	52.4	52.4
0.8	55.0	52.7	53.0	52.7
0.9	59.2	55.4	58.0	56.7
0.925	62.0	57.0	61.5	60.4
0.95	67.8	60.7	66.9	65.7
0.975	80.5	70.5	75.0	73.5
0.99	93.2	85.0	85.4	82.7

SUMMARY

Electrophoretic examination has shown that the $S_{14.6}$ and $S_{9.0}$ species have surprisingly similar mobilities which accounts for poor resolution in mixed systems. The general picture of the arachin dissociation (from Part I) concerning both equilibrium position and reaction rates has been confirmed for phosphate and barbiturate buffers.

The $SO_4^=$ ion is strongly adsorbed on the arachin system causing increased (-ve) mobilities for both components. Such ions are not removed by prolonged dialysis but are replaced by diethylbarbiturate ions. It is difficult to reconcile the adsorption of the $SO_4^=$ ion with its effect on the dissociation process.

On dilution of the arachin system, certain changes in electrophoretic diagrams occur which have no counterpart in sedimentation. Such changes have made it impossible to test electrophoretic anomaly theory experimentally as it concerns component areas, but the general lines of the theory have been confirmed by mobility measurements.

The experimental work of FONTAINE *et al.* has been largely confirmed but a different interpretation is offered.

RÉSUMÉ

L'examen électrophorétique a montré que les espèces $S_{14.6}$ et $S_{9.0}$ ont des mobilités étonnamment semblables ce qui explique la faible séparation obtenue avec des systèmes mixtes. L'aspect général de la dissociation de l'arachine (voir la 1ère partie de ce mémoire) en ce qui concerne la position de l'équilibre et les vitesses de réaction a été confirmé pour le cas des tampons phosphate et barbiturate.

L'ion $SO_4^=$ est fortement adsorbé par le système de l'arachine augmentant ainsi les mobilités des deux composantes. Ces ions ne sont pas enlevés par dialyse prolongée mais sont remplacés par les ions diéthylbarbituriques. Il est difficile d'accorder l'adsorption des ions $SO_4^=$ avec leur action sur le processus de dissociation.

Après dilution du système arachine on observe certains changements sur les diagrammes électrophorétiques qui n'ont pas de parallèle en la sédimentation. Ces changements ont rendu impossible la vérification expérimentale de la théorie de l'anomalie électrophoretique mais la theorie a pu être confirmée en ligne générale par des mesures de mobilité.

Nous avons confirmé en grande partie les travaux expérimentaux de FONTAINE *et coll.* mais nous présentons une interprétation différente.

References p. 396.

ZUSAMMENFASSUNG

Eine elektrophoretische Prüfung hat gezeigt, dass die Arten $S_{14.6}$ und $S_{9.0}$ erstaunlich ähnliche Mobilitäten besitzen, was die schwache Auflösung in Mischsystemen erklärt. Das allgemeine Bild der Arachindissoziation (siehe Teil I), was die Lage des Gleichgewichtes und die Reaktionsgeschwindigkeiten betrifft, wurde für Phosphatpuffer und Barbituratpuffer bestätigt.

SO_4-Ionen werden stark am Arachinsystem adsorbiert und erhöhen so die Mobilität beider Komponenten. Solche Ionen werden durch andauernde Dialyse nicht entfernt aber durch Diäthylbarbitursäure-Ionen ersetzt. Es ist schwierig die Adsorption der Sulfationen mit ihrer Wirkung auf den Dissoziationsprozess in Einklang zu bringen.

Bei Verdünnung des Arachinsystems treten im Elektrophoresediagramm Veränderungen auf, bei der Sedimentation ist hingegen nichts Entsprechendes zu beobachten. Diese Veränderungen vereiteln die experimentelle Prüfung unserer Theorie von der elektrophoretischen Anomalie. Diese Theorie wurde aber in grossen Zügen durch Mobilitätsmessungen bestätigt.

Die experimentelle Arbeit von FONTAINE *et al.* wurde grösstenteils bestätigt aber eine andere Interpretation vorgeschlagen.

REFERENCES

[1] T. D. FONTAINE, G. W. IRVING, AND R. C. WARNER, *Arch. Biochem.*, 7 (1945) 475; 8 (1945) 239.
[2] P. JOHNSON AND E. M. SHOOTER, *J. Colloid Sci.*, 3 (1948) 539.
[3] P. JOHNSON AND F. J. JOUBERT (1949), unpublished experiments.
[4] A. E. ALEXANDER AND P. JOHNSON, *Colloid Science*, Oxford Univ. Press, Vol. I (1949) p. 303.
[5] D. C. HENRY, *Proc. Roy. Soc.*, A 133 (1931) 106.
[6] H. A. ABRAMSON, L. S. MOYER, AND M. H. GORIN, *Electrophoresis of Proteins*, Reinhold (1942) p. 158.
[7] M. H. GORIN, unpublished calculations mentioned in Ref. 6, p. 131.
[8] H. SVENSSON, *J. Biol. Chem.*, 139 (1941) 805.
[9] L. G. LONGSWORTH, *J. Phys. Colloid Chem.*, 51 (1947) 171.
[10] V. P. DOLE, *J. Am. Chem. Soc.*, 67 (1945) 1119.
[11] H. HOCH, *Biochem. J.*, 42 (1948) 181.

Received December 24th, 1949